Using Near Infrared (NIR) Spectroscopy and Statistical Methods to Distinguish the Effects of Genetic and Environmental Variables on the Chemical Composition of Mature Corn Stover

Charnell Clarke
Office of Science, United Negro College Fund
University of the District of Columbia
National Renewable Energy Laboratory
Golden, Colorado

August 9, 2002

Prepared in partial fulfillment of the requirements of the Office of Science, DOE, and United Negro College Fund Special Program under the direction of DR. STEVEN R. THOMAS and TAMMY KAY HAYWARD in the Biotechnology Division for Fuels and Chemicals, National Renewable Energy Laboratory, Golden, Colorado 80401.

Participant:_____
Signature

Research Advisor:_____
Signature

Research Advisor:_____
Signature

Table of Contents

Using Near Infrared (NIR) Spectroscopy and Statistical Methods to Distinguish the Effects of Genetic and Environmental Variables on the Chemical Composition of Mature Corn Stover.

CHARNELL CLARKE (University of the District of Columbia, Washington DC 20008), DR. STEVEN R. THOMAS and TAMMY KAY HAYWARD (Biotechnology Division for Fuels and Chemicals, National Renewable Energy Laboratory, Golden, Colorado 80401).

Abstract. Ethanol can be used as an alternative fuel instead of gasoline or as an additive to gasoline in internal combustion engines. Almost all ethanol used in the United States today is derived from corn grain but corn stover (leaves, stem and cobs) is another potential renewable source of fermentable sugars for the production of ethanol that has not yet been commercialized. The chemical composition of corn stover is a large component in determining the economic viability of ethanol production from corn stover and other lignocellulosic feed stock materials. We are interested to determine the range over which the chemical composition of corn stover can vary and to what extent this variation is a function of genetic and environmental influences. Samples of several hybrids of corn stover grown under four different cultivation regimes at a single geographic location near Lincoln, Nebraska during the summer of 2001 were kindly supplied by the USDA/ARS, Lincoln, NE. Cultivation conditions varied as follows: Irrigation vs. no irrigation (rain only) and fertilizer (200 lbs nitrogen/ acre) vs. no fertilizer. Replicate near-infrared (NIR) reflectance spectra were collected from two different sub-samples of each of the ninety-six stover samples collected. Raw NIR data were interpreted using a mathematical model to predict the chemical composition of the sample (dry weight basis). The predicted chemical compositions were analyzed for correlations with the known genetic and environmental variables.

Research Category: Environmental Sciences

School Author Attends: University of the District of Columbia
DOE National Laboratory Attended: National Renewable Energy Laboratory

Mentors Names:	Dr. Steven Thomas	Tammy Kay Hayward
Phone:	303-384-7775	303-384-7784
E-mail Address:	Steven_Thomas@nrel.gov	Tammy_Hayward@nrel.gov

Presenter's Name: Charnell Clarke
Mailing Address: 5022 Illinois Ave. NW
City/State/Zip: Washington DC 20011
Phone: 202-829-4137
E-mail Address: Dimples1444@aol.com
Is this being submitted for publication? Yes

DOE Program: UNCF

Introduction

Biomass including corn (*Zea mays L.*) grain has been used for many years as a source of renewable energy. This energy in the form of ethanol has been used as fuel in internal combustion engines. In 1908 Henry Ford designed his model T to run on alcohol partly because it worked and partly because it would benefit the farm industry (Wiselogel, 1998). The American industry drifted from the production of ethanol as fuel because it was more efficient to produce gasoline from refined crude oil, but ethanol use bounced back as a cleaner burning octane enhancer that replaces lead in gasoline. The U.S. Department of Energy projects that demand for ethanol will increase to 3.5 billion gallons per year by 2010 due to the nation's interest in reducing its dependency on foreign energy through domestically produced renewable energy sources that improve our air quality and reduce global warming (BBI International, 2001).

With this increased demand for ethanol, the focus for renewable methods of production is of concern. Ethanol can be produced from non-grain plant materials such as corn stover. Corn stover (cobs, stalks, and leaves) has no human food value, although it is sometimes fed to cattle. Currently, most stover is plowed back into the fields to enrich the soil and prevent erosion. In a no-till environment, significant quantities of corn stover can be sustainably harvested. If the corn processing industry is willing to take advantage of this abundant feedstock, it can reduce the volume of agricultural waste and produce much greater quantities of fuel grade ethanol (Wiselogel, 1998).

In order for ethanol to be economically produced from corn stover, the cell wall of the corn stover must have a high concentration of carbohydrates (cellulose and hemicellulose) that can be hydrolyzed with the aid of acid and enzymes into sugars (glucose and xylose). A fungus (yeast) or bacteria then ferment these sugars to produce ethanol.

The rapid analysis method employed to determine the cell wall composition of corn stover is a Near-Infrared (NIR) FOSS Forage Analyzer machine. This machine scanned corn stover that had been milled to pass through a ¼ inch mesh screen. Each sample of corn stover that was placed in the NIR machine was scanned thirty times and the average of those sums were stored on the computer. The calibrated corn stover model then predicted the percent of the dry weight composition for all components.

The intention of this investigation was to determine the range over which the chemical composition of corn stover can vary and to what extent this variation was a function of genetic and environmental influences. The environmental conditions under which these samples of corn stover were cultivated were examined for this study. These cultivation conditions varied as follows: Irrigation vs. no irrigation (rain only) and fertilizer (200 lbs nitrogen/acre) vs. no fertilizer.

The water requirement for corn depends on the corn variety and the rate of evapotranspiration. Long-season corn varieties use more water but they also produce more grain. Corn is more sensitive to water stress than other field crops and does not

extract water uniformly throughout the rooting depth (Benham, 1998). Also corn contains more nitrogen in its grain than any other soil-derived nutrients and substantially more nitrogen fertilizer is used for its production than other primary fertilizer nutrients. Nitrogen is by far the most transitory of all fertilizer nutrients being subject to losses from both leaching and microbial oxidation. Most nitrogen is absorbed into corn roots through mass flow of NO_3^-, a highly soluble ion that moves in the direction of moisture flow (Sprague and Dudley, 1988). With this in mind, the data will be analyzed for any impact of these environmental factors on the cell wall composition of the corn stover.

In the long run, the ultimate goal for researching cell wall composition of corn stover is to make the production of ethanol from corn stover economically viable. Cellulose and hemicellulose improvements are aspects of the ethanol-from-biomass process with great potential for cost reduction. If this is done successfully, a whole new industry could evolve from the ability to produce chemicals and materials from renewable biomass instead of fossil fuels (Brown, 1993).

Materials and Methods

Source of Corn Stover Materials

A grain yield trial called "Pioneer Fluorescence Study MSEA" was conducted in Lincoln, Nebraska during the summer of 2001. One hybrid (B73 x MO17) and nine Pioneer commercial hybrid varieties (3417, 33R88, 34K78, 3162, 34G82, 3394, 33A14, 33G27, and 34D34) of corn were grown with and without fertilizer and with and without irrigation. One hundred and forty four corn plants were grown in a complete design.

These were planted serpentine in three replicate plots. Dr. Wally Wilhelm (USDA/ARS, Lincoln, NE) only supplied ninety-six corn stover samples taken from random areas in the complete design sample set (see table 1). These ninety-six samples grown under different genetic and environmental conditions were sent to NREL for inclusion in this study.

Processing of Corn Stover

Fully mature corn stover was harvested by hand after grain harvest, dried completely at 50°C for 24-72 hours, shipped to NREL by common carrier in cardboard boxes, and stored at room temperature. On arrival at NREL, each sample was assigned a unique identifying sample number. A contractor (Hazen Research, Inc., Golden, CO) individually milled corn stover samples to pass through a ¼ inch mesh screen. The contractor also riffle-split each milled stover sample into equivalent 500 g aliquots and placed them in labeled plastic zip-lock bags for storage at room temperature in labeled plastic buckets.

Near-Infrared (NIR) Spectroscopy

Two sub-samples of milled bulk corn stover were taken from a single zip-lock bag and placed into two identical natural product cells for Near-Infrared (NIR) spectroscopy. The contents of each cell were gently leveled off and the cell closed. After the quartz surface of the cell was wiped clean of any particles that could interfere with the NIR scanning process, the cell containing the bulk corn stover was loaded in the FOSS Forage Analyzer. The spectrometer scanned each sample thirty times and averaged them to

produce a single stored data file. The spectrometer produced NIR spectra in the wavelength range between 400 and 2500nm. A calibrated mathematical model then calculated the chemistry of the sample from the NIR spectrum for each sample, which includes eleven chemical constituents, including structural glucan, xylan and lignin. The output data will be combined with the original grain yield data (not yet received) for statistical analysis of genetic and environmental effects on the chemical composition of the mature corn stover.

Statistical Analysis

Graphs and tables were used to illustrate trends. A 2^2 Full Factorial Design method was employed to derive trends and correlations in the data. For the factorial method a sub sample of thirty-two plants from replicate 1 (see table 1) was used to derive a full 2^2 design. These thirty-two samples were sorted into four groups that would represent the environmental conditions being examined. The groups were; fertilized and irrigated, not fertilized but irrigated, fertilized but not irrigated, and not fertilized and not irrigated. Analysis was also done for the variety effect and soil effects as replicate plots.

Results

The FOSS NIR spectrometer predicts the chemical composition of all corn stover samples it scanned. There are models for several types of material built into the system against which the spectrometer is calibrated. Bulk corn stover is one of these materials for which the spectrometer can accurately determine the chemical composition. The chemical cell wall constituents that the FOSS machine determines for corn stover are

structural glucan, soluble glucose, xylan, lignin, protein, acetyl, uronic acid, galactan, arabinan, mannan, structural inorganics, and soil. Galactan, arabinan, and mannan are minor sugars that the FOSS machine will predict but they do not play any significant role in these results because they are only significant if there is a high range in their content. Because of the errors associated with determining the chemical composition by wet chemistry, there is a built-in method error of +/- 1.5% by dry weight in the FOSS NIR machine. Therefore if the range is within 3%, the samples are the same. Figure 1 is a pie chart indicating the average chemical composition of the ninety-six corn stover samples as determine by the FOSS machine.

The computer also determines the global H and neighborhood H values. The global H value indicates how well the sample composition fits the model. A global H value of 3 or more indicates that the data doesn't fit the model well. The neighborhood H indicates the proximity of the particular sample to the other samples within the method. The maximum global H value for these samples was 2.8 and the maximum neighborhood H value was 1.5. Unless the method over or under predicts a constituent, the mass closure should be near 100%. For the ninety-six samples studied the average mass closure value was 97.4%. Table 2 contains a summary of this information.

Of the ninety-six-corn stover samples studied, the dry weight composition of the cell wall ranged from a high of 44.6 % total glucan to a low of 38.6 % and for protein, a high of 5.6% to 2.0% (see table 2). All of the samples studied fall within 2 standard deviations of the mean i.e. 95% confidence interval. The mean and standard deviation for all the

samples are given in table 2. Table 3 represents the effect of the three different replicate plots of soil on the chemical composition of the corn stover. Regression graphs for the 96-stover samples were done for the major sugars and labeled accordingly. Figures 2 through 9 are examples of the result obtained from those graphs.

From the graphs of these major sugars, it was clear that there were no strong relationship between the constituents. Lignin vs. structural glucan had the most impressive result. There was a positive relationship between them and the data fit the regression line better than the other pairs of constituents (see figure 5). Lignin v structural glucan had a R^2 value of 0.6. This is only a fair correlation value because it should be closer to 1.0 for a perfect correlation. However compared to the other values, which were 0.5 and below, it was the strongest correlation. Structural inorganics vs. structural glucan had the poorest fit to the regression line. The data spread was almost circular with a R^2 value of 0.06.

In the 2^2 factorial design method of analysis, thirty-two random samples were taken from replicate 1, as this was the only complete data set supplied by Dr. Wally Wilhelm. Table 4 represents this data. The results for the sub-sample used for this method of analysis are given in tables 5, 6, and 7 with respect to the effects of fertilizer, irrigation and any interaction.

Samples grown in replicate 2 and 3 under the different environmental conditions that were received were added into the 2^2 factorial design method. An average of the chemical components for each environmental condition was determined. Then the

average of each environmental condition for each variety was taken. This was done in order to evenly weight the effect of all environmental conditions on the particular variety. The effect on the different varieties is given in table 8.

Discussion and Conclusion

The ninety-six samples of corn stover were planted in the same region of Lincoln, Nebraska. They were planted on three replicate plots in a serpentine order. The three different replicate plots of soil in which the corn was planted had no effect on the chemical composition of the corn stover. The results were within the +/- 1.5% error associated with the method (see table 3).

The maximum global H of 2.8 means that all of the samples are within the tolerance of the model. Therefore the compositions of these samples are trustworthy and accurate. This was expected because the model was built on samples from commercial Pioneer hybrids and inbreeds. The samples in this study are mostly commercial Pioneer varieties and are within the limits of the wet chemical methods on which the NIR model is based. The maximum neighborhood H value was 1.5 and this proves even more that the data was trustworthy because the samples studied were a very close match to groups of samples within the model. The global H and neighborhood H values are significant because it shows that the computer model did not extrapolate values for these samples and that the model was able to accurately predict the cell wall composition.

Due to a cumulative effect of wet chemical errors in each of the 12 constituents, the expected experimental error for mass closure is +/- 5%. From the data, the average mass closure was calculated to be 97.4%. This also substantiates that the NIR model is predicting correctly because the mass of the samples were taken into account. On the other hand, if the mass closure value were 70%, the model might have under predicted one or more of the chemical components of the sample. Also a mass closure value of 120% would suggest that the computer is over-predicting one or more chemical component and the results could not be trusted.

Acetyl, uronic acid, galactan, arabinan, mannan, and soil varied within the +/-1.5% error in the entire data set. There was no statistically significant variability among these samples for these constituents. Therefore fertilizer and irrigation effects could not be determined for these constituents. However, structural glucan, soluble glucose, xylan, protein, lignin, and structural inorganics were further analyzed because there were significant differences in their values from sample to sample.

The results from the 2^2 factorial design experiment did not indicate any statistically significant findings for the environmental influences tested. However, structural glucan and soluble glucose had the greatest difference as a result of being fertilized (see table 5). Xylan, lignin, and structural inorganics varied the greatest as a result of irrigation (see table 6) but again these observations are not regarded as statistically significant. There was no interaction effect between fertilizer and irrigation because there was no difference in the chemical components (see table 7). Analysis of genetic effects showed that

Pioneer 34G82 and Pioneer 33A14 had the greatest difference in structural glucan content. The 4.0% difference in structural glucan is greater than the method error of +/- 1.5% and is therefore significantly different. This indicates that genetics had an effect on the chemical composition of the corn stover. Variety 33A14 had the greatest amount of carbohydrate as well (see table 8).

Based on these results there are no significant effects of the studied range of fertilizer and irrigation on the cell wall chemical composition of corn stover grown in this geographic location. However, this is promising for the economics of the ethanol process. This study has begun to identify two variables that will not affect the chemical composition of corn stover grown in this particular area (Lincoln, NE). Farmers will not have to be concerned about the amount of fertilizer or amount of water corn plants received in order to produce a relatively constant quality corn stover for the ethanol process. The samples were taken from different plots of the same field so the soil effect was negligible (see table 3).

Looking at the economics of the ethanol process, cost can be kept to a minimum because there is no additional cost for fertilizer, water or land. Pioneer variety 33A14 had the highest structural glucan content as well as the highest carbohydrate content and can be recommended as one of the better varieties to plant in order to produce corn stover with high carbohydrate content. High carbohydrate content is important because it is the carbohydrates that are fermented into ethanol. Also the more carbohydrates present in the corn stover, the more ethanol will be produced.

Further work needs to be done in order to fully understand the genetic and environmental variables that are affecting the cell wall chemical composition of the corn stover. Corn stover need to be collected from different geographic locations and the temperature and day length analyzed. The pH and mineral content of the soil need to be examined for its effect on the cell wall chemical composition. Also further research should be conducted to look at varying the amount of irrigation as well as looking into genetically engineered varieties and their effect on the chemical composition of the cell wall in the corn stover.

Acknowledgement

I would like to thank the United States Department of Energy for providing me with this opportunity to gain invaluable knowledge and experience in the field of biotechnology. Thanks also to the National Renewable Energy Laboratory and its employees for taking the time to help me through this learning experience. I thank my mentor, Dr. Steven Thomas especially for sharing his knowledge of corn and biomass technology with me. Special thanks also to Tammy Kay Hayward for assisting me with my research project even when I was getting frustrated. I would like to thank Amie Sluiter for teaching me how to use the various machines and Liz Wilson for her caring support. Thanks to Laverne Means at the United Negro College Fund Special Programs who worked so hard in making it possible for me to come out to Colorado on such short notice. Thanks also to Linda Lung and her staff for their constant support and supervision and for making this internship truly a learning experience.

References

BBI International. (2001). *The U.S. Dry-Mill Ethanol Industry Biobased Products and Bioenergy Initiative Success Stories*. Golden, CO: National Renewable Energy Laboratory.

Benham, Brian L. (1998). Irrigating Corn [Electronic Version]. *NebGuide* (pp. 2-4). Lincoln, NE: Institute of Agriculture and Natural Resources.

Brown, Howard. (1993). *Mini-Manhattan Project for Cellulases Research Brief*. Golden, CO: National Renewable Energy Laboratory.

Sprague, G.F., Dudley, & J.W. (Eds.). (1988). *Corn and Corn Improvement* (pp.665-666). (3rd ed.). Madison, WI: The American Society of Agronomy.

Wiselogel, Art. (1998). *Bioethanol from the Corn Industry*. Golden, CO: National Renewable Energy Laboratory.

Table 1: Samples not received from field grain study shown in black.

101	3394	124	3394	125	3394	148	3394
	33H67		33H67		33H67		33H67
	3162		3162		3162		3162
Irr	33R88	Irr	33R88	Dry	33R88	Dry	33R88
	33G27		33G27		33G27		33G27
	34K78		34K78		34K78		34K78
	34G82		34G82		34G82		34G82
Fert	34D34	No Fert	34D34	No Fert	34D34	Fert	34D34
	3417		3417		3417		3417
	34R07		34R07		34R07		34R07
	B73/Mo17		B73/Mo17		B73/Mo17		B73/Mo17
112	33A14	113	33A14	136	3A14	137	33A14
201	34D34	224	34D34	225	34D34	248	34D34
	33G27		33G27		33G27		33G27
	34R07		34R07		34R07		34R07
Dry	3417	Dry	3417	Irr	3417	Irr	3417
	3162		3162		3162		3162
	33A14		33A14		33A14		33A14
	34K78		34K78		34K78		34K78
No Fert	33H67	Fert	33H67	No Fert	33H67	Fert	33H67
	3394		3394		3394		3394
	B73/Mo17		B73/Mo17		B73/Mo17		B73/Mo17
	34G82		34G82		34G82		34G82
212	33R88	213	33R88	236	33R88	237	33R88
301	34R07	324	34R07	325	34R07	348	34R07
	33A14		33A14		33A14		33A14
	33H67		33H67		33H67		33H67
Irr	34G82	Irr	34G82	Dry	64G82	Dry	34G82
	33G27		33G27		33G27		33G27
	3162		3162		3162		3162
	34D34		34D34		34D34		34D34
Fert	B73/Mo17	No Fert	B73/Mo17	Fert	B73/Mo17	No Fert	B73/Mo17
	33R88		33R88		33R88		33R88
	3417		3417		3417		3417
	34K78		34K78		34K78		34K78
312	3394	313	3394	336	3394	337	3394

N

Rep 1

Rep 2

Rep 3

Plots number serpentine within reps from the Northwest corner (south, then east).

Code	
Border	Grey
Not irrigated, no N	White
Irrigated, no N	Yellow
Not irrigated, 200 kg N ha^{-1}	Pale green
Irrigated, N 200 kg N ha^{-1}	Dark green
Samples not received	Black

Figure 1: Average Corn Stover Composition in USDA Set.

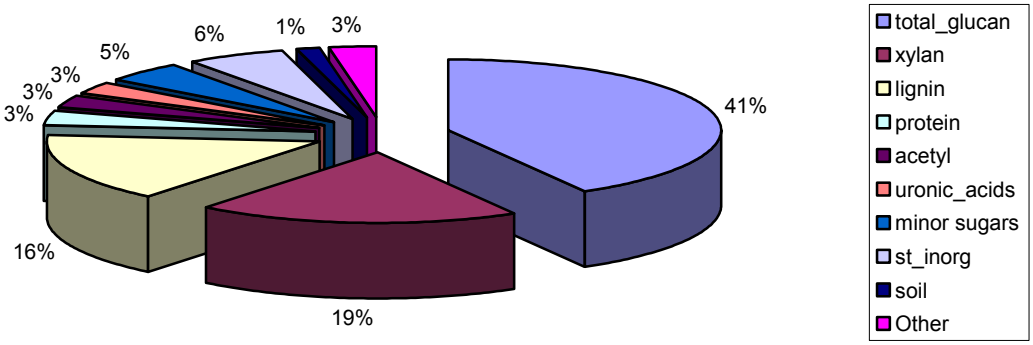

Table 2: Descriptive statistic of the 96 Corn Stover Samples.

Components	MAX	MIN	RANGE	MEAN	STDEV	COUNT
total_glucan	44.6	38.1	6.5	41.7	1.2	96
struct_glucan	38.8	30.0	8.9	35.5	1.8	96
Soluble Glucose	9.3	3.4	5.8	6.2	1.4	96
xylan	21.6	15.2	6.3	19.0	1.6	96
lignin	17.9	12.4	5.5	15.8	1.3	96
protein	5.6	2.0	3.5	3.2	0.7	96
acetyl	3.5	2.2	1.3	2.9	0.2	96
uronic_acids	3.2	2.1	1.1	2.8	0.2	96
galactan	2.0	1.0	1.0	1.6	0.2	96
arabinan	2.9	1.7	1.2	2.4	0.3	96
mannan	1.1	0.0	1.0	0.5	0.2	96
st_inorg	10.2	2.2	8.0	6.0	1.7	96
soil	1.7	1.3	0.4	1.5	0.1	96
Mass Closure	100.4	91.8	8.6	97.4	1.8	96
Global H	2.8	0.7	2.1	1.6	0.5	96
Neighborhood H	1.5	0.3	1.2	0.7	0.3	96

Table 3: No effect on average corn stover composition in the 3 replicate soil plots.

Plots	%Structural Glucan	% Xylan	%lignin
Replicate 1	35.4	19.5	16.0
Replicate 2	35.8	18.9	15.9
Replicate 3	35.	18.7	15.4
Maximum Difference	0.5	0.8	0.6

Figure 2: No correlation between lignin and protein.

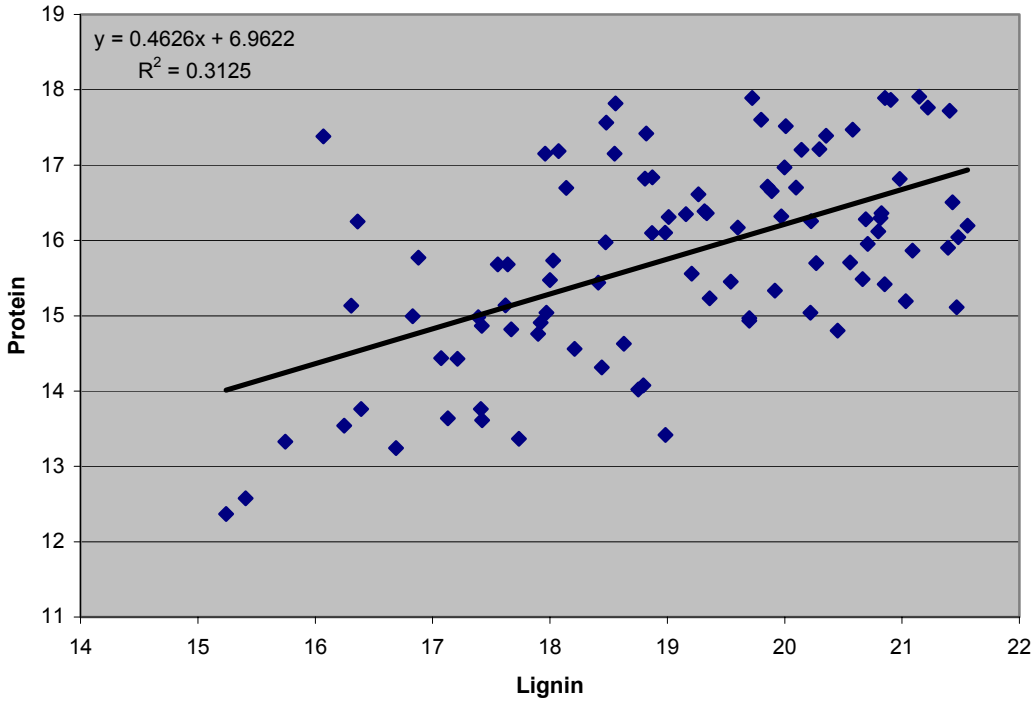

Figure 3: No correlation between structural inorganics and protein.

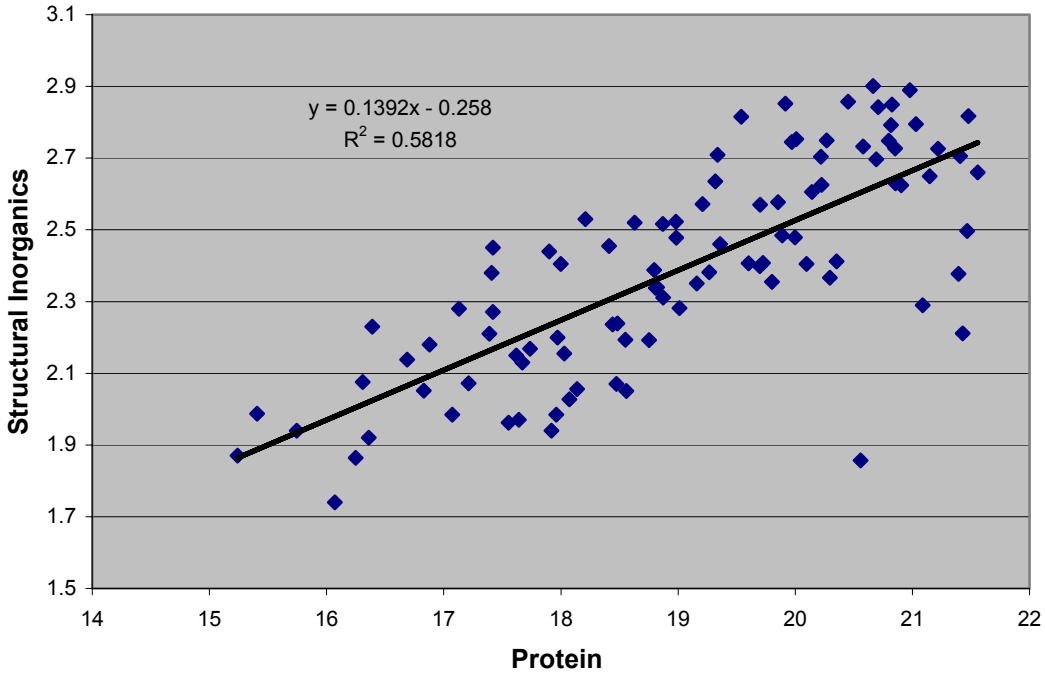

Figure 4: No correlation between protein and xylan.

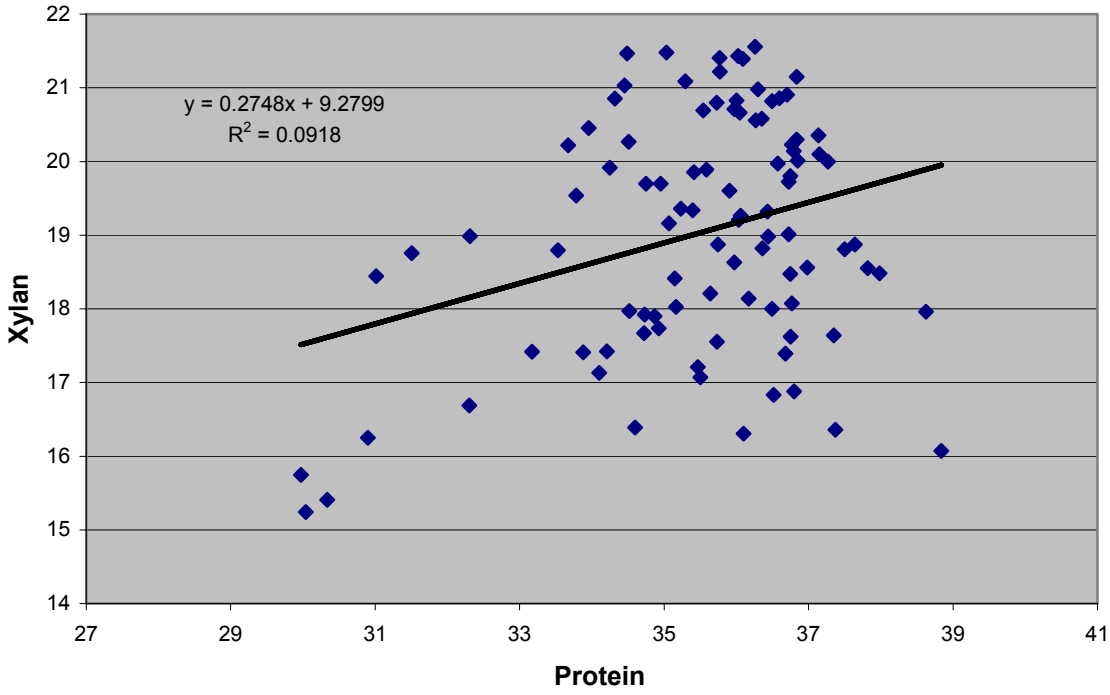

Figure 5: No correlation between lignin v structural glucan

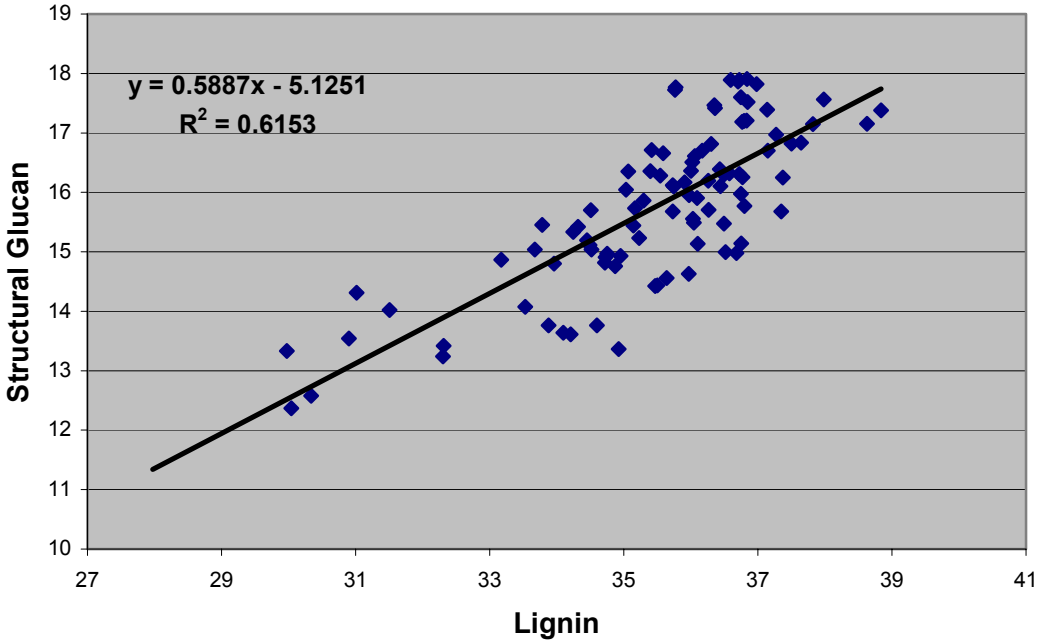

Figure 6: No correlation between structural glucan and protein

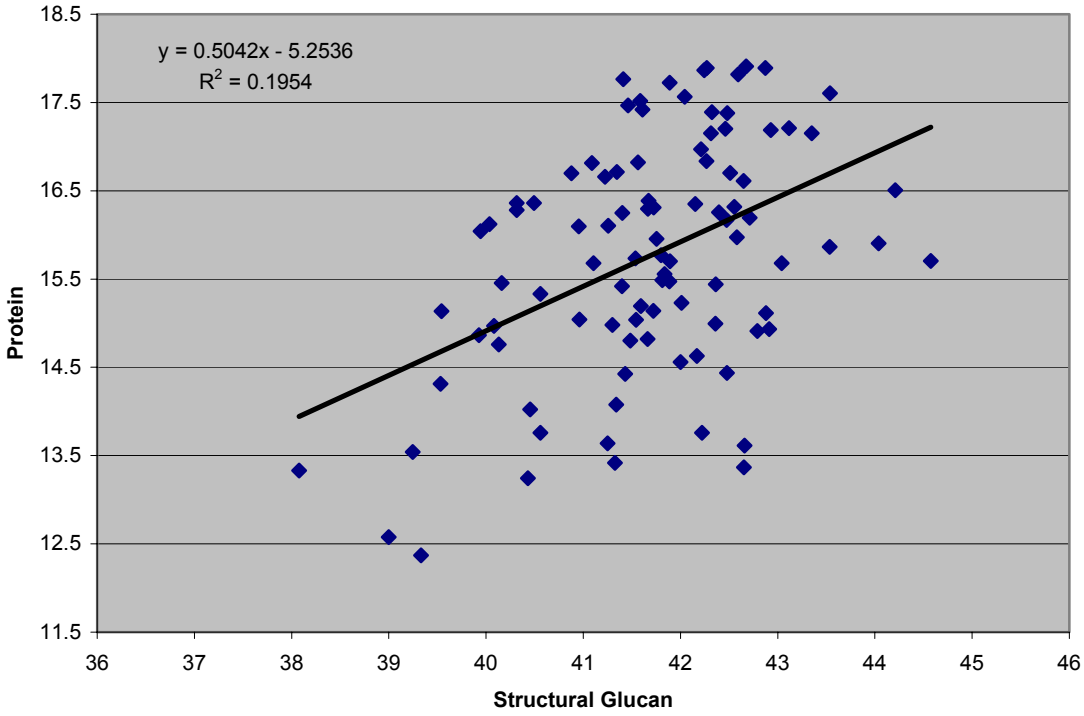

Figure 7: No correlation between structural inorganics and structural glucan

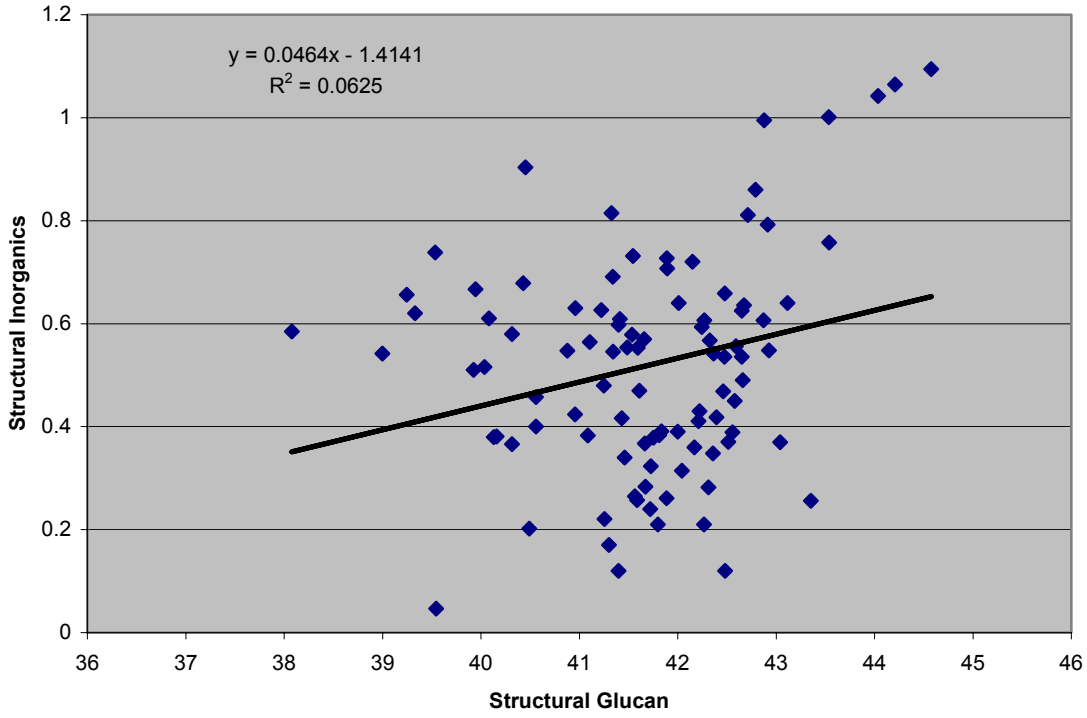

Figure 8: No correlation between structural inorganics and xylan

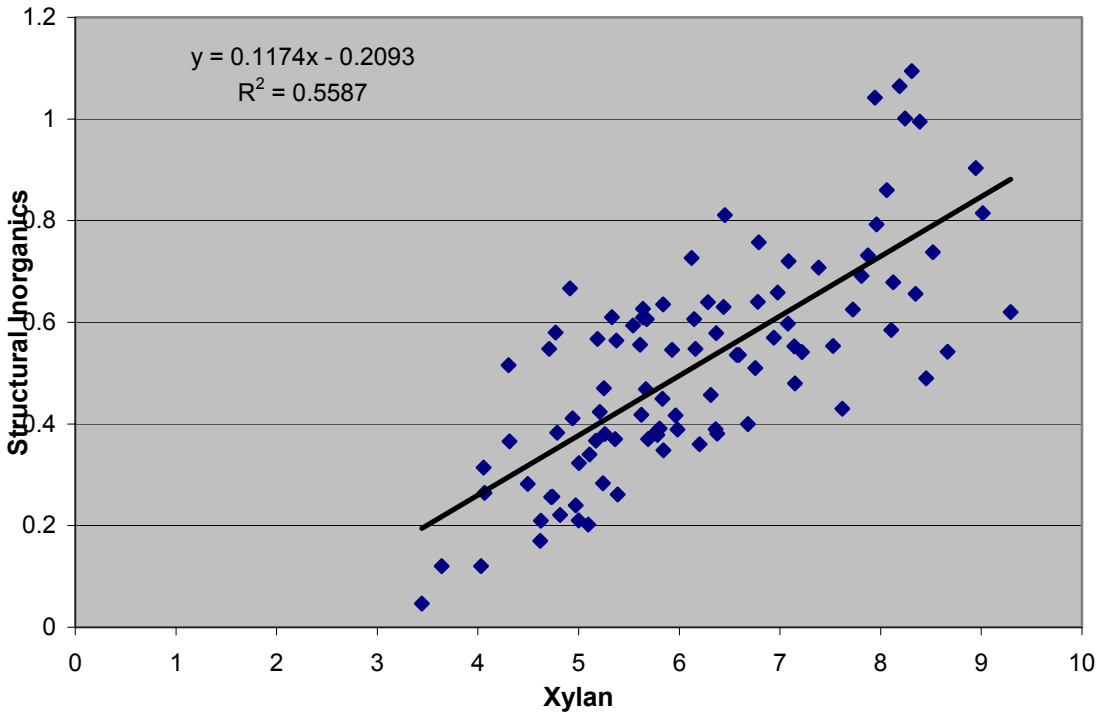

Figure 9: No correlation between lignin and structural inorganics

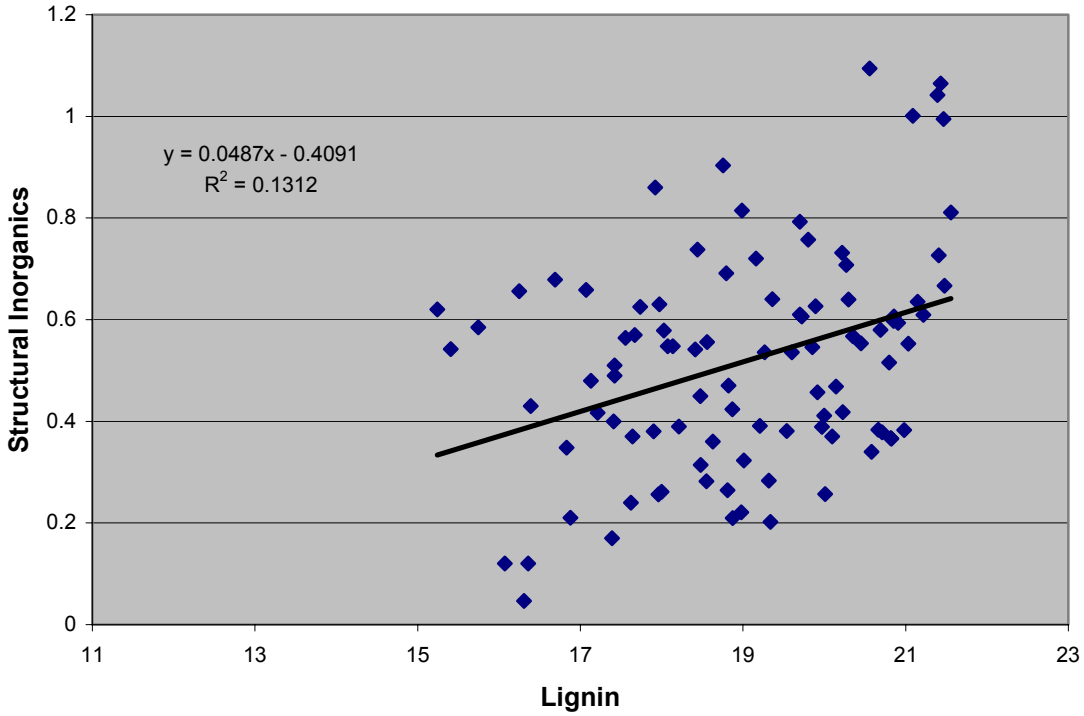

Table 4: Varieties not received and not used in full 2^2 Factorial Design in black

101	3394	124	3394	125	3394	148	3394
	33H67		33H67		33H67		33H67
	3162		3162		3162		3162
Irr	33R88	Irr	33R88	Dry	33R88	Dry	33R88
	33G27		33G27		33G27		33G27
	34K78		34K78		34K78		34K78
	34G82		34G82		34G82		34G82
Fert	34D34	No Fert	34D34	No Fert	34D34	Fert	34D34
	3417		3417		3417		3417
	34R07		34R07		34R07		34R07
	B73/Mo17		B73/Mo17		B73/Mo17		B73/Mo17
112	33A14	113	33A14	136	3A14	137	33A14

N

Rep 1

Table 5: Analysis of potential effects of fertilizer addition

Environmental Conditions	% Structural Glucan	% Xylan	% Lignin
Fertilized	34.6	19.2	15.7
Not Fertilized	36.2	19.8	16.2
Maximum Difference	1.5	0.6	0.5

Table 6: Analysis of potential effects of irrigation

Environmental Conditions	% Structural Glucan	% Xylan	% Lignin
Irrigated	35.5	19.8	16.2
Not Irrigated	35.3	19.2	15.7
Maximum Difference	0.2	0.6	0.5

Table 7: Analysis of potential effects of interaction between fertilizer and irrigation

Environmental Conditions	% Structural Glucan	% Xylan	% Lignin
Fertilizer	35.5	19.6	16.0
Irrigation	35.3	19.4	15.9
Maximum Difference	0.2	0.2	0.1

Table 8: Analysis of potential effects of genetics

Hybrid/Inbred	struct_glucan	xylan	lignin	protein	st_inorg	carbohydrate	# Of samples
3417	36.5	19.8	17.0	2.9	4.9	56.3	11
B73/MO17	36.3	18.8	16.4	3.1	5.7	55.1	12
33R88	35.4	19.0	15.3	3.2	6.2	54.4	12
34K78	35.9	18.3	15.8	3.1	6.5	54.3	11
3162	35.0	18.9	15.7	3.3	6.3	53.9	12
34G82	33.1	18.2	14.3	4.0	7.2	51.3	13
3394	36.1	18.9	15.8	3.0	6.1	55.0	12
33A14	37.1	20.1	16.7	2.8	4.9	57.2	1
33G27	35.9	20.6	15.9	2.9	4.4	56.4	11
34D34	35.4	19.3	16.4	3.2	6.6	54.7	1
Maximum	37.1	20.6	17.0	4.0	7.2	57.2	96
Minimum	33.1	18.2	14.3	2.8	4.4	51.3	
Difference	4.0	2.4	2.7	1.2	2.8	5.9	